THE EFFECT OF SULPHASALAZINE ON NEUTROPHIL SUPEROXIDE GENERATION IN RHEUMATOID ARTHRITIS

S. M. BRADLEY, P. LE GALLEZ,* P. R. TROUGHTON, H. C. GOOI,† C. ASTBURY‡ and H. A. BIRD

Clinical Pharmacology Unit (Rheumatism Research), University of Leeds, Chapel Allerton Hospital, Chapeltown Road, Leeds LS7 4SA and *St James’s University Hospital, Beckett Street, Leeds LS7 9TF

SUMMARY

The production of superoxide by the peripheral blood neutrophils of 19 patients with active rheumatoid arthritis was measured during treatment with sulphasalazine (SASP). The response to drug treatment was determined by change in plasma viscosity, CRP, early morning stiffness and articular index over a 10-point scale. Of the 19 patients studied, eight were considered to have responded well to SASP and seven to have responded poorly or not at all. Over the treatment period, plateau levels of superoxide production fell in seven of the eight responders (P = 0.028) compared with a non-significant fall in 3/7 of the non-responder groups. The initial rate of superoxide production also fell in the responder group, but this was not statistically significant. Initial values in both the responder and non-responder groups were comparable with those seen for normal controls. Analysis of drug levels showed all patients to be compliant with drug treatment; however, drug levels and neutrophil activity were not correlated. Studies of the effect of SASP and sulphapyridine on superoxide production in vitro showed no difference between good and poor responders. These results suggest that there is no inherent difference between good and poor responders regarding the susceptibility of their neutrophils to SASP. SASP’s action on neutrophils, therefore, appears not to be its main mechanism of disease-modifying activity in RA.

KEY WORDS: Sulphasalazine, Response, Neutrophils, Superoxide.

ALTHOUGH sulphasalazine (SASP) is an effective second-line agent in the treatment of rheumatoid arthritis (RA), 24–39% of treated patients withdraw due to lack of efficacy [1, 2]. This may be because of the several genetic and acquired factors that influence drug metabolism or due to differences in the susceptibility of various components of the immune system to the drug. Factors that have been investigated so far include gender, disease duration, acetylator status, previous second-line treatment, and serum levels of the drug and its major metabolites [3–5].

Of the ingested SASP, <30% is absorbed intact in the small intestine, the remainder being cleaved in the colon by bacteria to liberate sulphapyridine (SP) and 5-aminosalicylic acid (5-ASA). The mode of action of SASP is unknown but, in contrast to ulcerative colitis, SP rather than 5-ASA appears to be the active compound, although a role for the parent molecule cannot be ruled out [6–8].

In addition to anti-microbial [9–11] and anti-folate [12] properties, SASP and SP display a wide range of anti-inflammatory and immunosuppressive actions which may account for their mode of action in RA. These include a reduction in the number of monocytes and B lymphocytes [13], serum IgA [14] and IgA-producing cells [15], and inhibition of leukotriene production [16], histamine release from peripheral leucocytes [17] and lymphocyte transformation [18, 19]. SASP and SP have also been shown to inhibit neutrophil chemotaxis [20] and superoxide production [21] elicited by N-formyl-methionyl-leucyl-phenylalanine.

Previous work in this unit has shown that levels of serum IgG antibody to an acetone powder of Clostridium perfringens were significantly higher in those patients who were regarded as having responded poorly to SASP [11]. This effect was not seen in a control group who responded well to treatment. The reason for this is unclear, but immune mechanisms may be involved. Clearly, the possibility that levels of serum antibodies to bacterial antigens or other aspects of the immune response may be used as markers for the likelihood of response to SASP could have important implications regarding the targeting of SASP therapy.

Neutrophils have an important role in the pathogenesis of RA via the release of lysosomal enzymes and reactive oxygen radicals. Given this and the effect SASP and SP have on neutrophil activity, we investigated neutrophil superoxide production in responders and non-responders to SASP. In addition, the effect of SASP and SP in vitro and in vivo on neutrophil superoxide production was studied to establish whether there were any differences in patients classified as good and poor responders to SASP in terms of the susceptibility of their neutrophils to drug therapy.

METHODS

Patients

Twenty-four patients with classical or definite RA (new ARA criteria) were recruited from the out-patient clinic at the General Infirmary at Leeds. All had active
determined spectrophotometrically by monitoring superoxide dismutase-inhibitable reduction of cytochrome c. Briefly, cytochrome c (0.625 μg/ml) was added to neutrophils (1 × 10⁶ cells/ml) in HEPES buffered Hank’s balanced salts solution (HBSS) containing calcium (1.5 mM) and magnesium (1.3 mM). Cells were stimulated with N-formyl-methionine-leucyl-phenylalanine (10 ng/ml) and the change in absorbance was measured continuously at 37°C in a Kontron Uvikon 930 spectrophotometer. Neutrophil superoxide generation was also measured in the presence of 10 μM SASP, 100 μM SP and 500 μM SP. These concentrations were chosen on the basis of being therapeutically relevant (10 μM SASP, 100 μM SP) and/or for giving ~50% inhibition in the assay (10 μM SASP, 100 μM SP). Trypan blue exclusion showed that these concentrations of SASP and SP did not affect cell viability. Results were expressed as either the initial linear rate of increase or plateau levels of superoxide production. The effect of SASP or SP was expressed as the percentage of superoxide produced by neutrophils compared to controls containing no SASP or SP. All assays were performed in duplicate.

**Response criteria**

Disease activity for each patient was assessed on day 0 of the study and at 4-weekly intervals thereafter for a maximum of 16 weeks using a number of clinical and laboratory assessments including PV, CRP, EMS and AI.

At each time point, the patients were given a score for each of the four variables, as indicated in Table I. The response to drug treatment was assessed by determining the change in score for each of these variables between weeks 0 and 16 of the study. Each patient was then given a total score for the four assessments. Patients with a score of >10 were classified as ‘good responders’. Those with a score of <4 were classified as ‘poor’ or ‘non-responders’.

**Neutrophil isolation and superoxide generation**

Neutrophils were isolated from patients’ peripheral blood on day 0 and weeks 8 and 16 of the study by dextran sedimentation, hypotonic lysis of red blood cells and Ficoll Hypaque density-gradient centrifugation [22]. Samples were also provided by nine healthy volunteers as controls. This procedure yielded preparations containing >95% neutrophils. Viability of the neutrophils was confirmed using trypan blue exclusion. Neutrophil superoxide generation was

**Statistics**

The Wilcoxon signed rank test was used to compare data at weeks 4, 8 and 16 with that of baseline values. The Mann–Whitney U-test was used to compare data at the same time point in the two groups of patients showing a good and poor response.

**RESULTS**

Of the 24 patients recruited to the study, four dropped out by week 4 due to side-effects. These were skin rash, mouth ulcer, severe nausea, depression and tinnitus. One patient failed to attend clinic after week 4. The results from these five patients were discounted from the study. Using the 10-point scale shown in Table I, eight patients were considered to have been good responders and seven patients poor responders. Those patients classed as responders showed a significant improvement in PV, CRP, rheumatoid factor (RF) and AI by week 8 of treatment, and EMS and pain score by week 16 (Table II). Those patients classed as poor responders showed only transient changes of significance in PV and AI at week 8 of treatment.

**TABLE I**

<table>
<thead>
<tr>
<th>Score</th>
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<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>PV (cP)</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
</tr>
<tr>
<td>Pain</td>
</tr>
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</table>
TABLE II
Changes in biochemical and clinical variables during SASP treatment in ‘good’ and ‘poor’ responders

<table>
<thead>
<tr>
<th>Study week</th>
<th>PV</th>
<th>CRP</th>
<th>A1</th>
<th>EMS</th>
<th>RF</th>
<th>Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>16</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results from poor responders are shown in parentheses. NS, not significant.

Superoxide production
At day 0 of the study, median plateau and initial rate levels of superoxide production in the 19 remaining patients were 0.186 [optical density (OD) at 550 nm] and 3.991 (nmol O₂/min/10⁶ cells), respectively, compared with values of 0.149 (OD 550 nm) and 2.881 (nmol O₂/min/10⁶ cells) in normal controls. At week 16 of the study, these were 0.187 (OD 550 nm) and 2.996 (nmol O₂/min/10⁶ cells), respectively, showing no significant change.

When the data for patients classified as either good or poor responders were analysed, plateau levels were shown to fall in seven out of eight of the good responders (Fig. 1), this being statistically significant ($P = 0.028$). This compared to a fall in only three out of seven of the poor responders. The changes in the median plateau levels were 0.196 to 0.141 (OD 550 nm, $P = 0.028$) in the responder group compared to 0.258 to 0.215 (OD 550 nm) in the non-responder group (not significant). Initial rate levels also fell from 4.379 to 2.567 (nmol O₂/min/10⁶ cells) in responders compared with a drop from 4.982 to 3.894 (nmol O₂/min/10⁶ cells) in poor responders, but this did not reach significance (Fig. 2). There was no difference in neutrophil activity between good and poor responders at either day 0 or week 16. In vitro studies of the effect of SASP and SP on neutrophil superoxide production also showed no difference between good and poor responders (Table III).

Although analysis of SASP, SP and acetylsulphapyridine (AcSP) levels in plasma showed compliance with drug therapy during the study, there was no significant correlation (Spearman rank correlation rho = 0.325, $P > 0.2$) between drug levels and neutrophil superoxide production during the treatment period (data not shown). Similarly, there was no significant difference in drug levels between the good and poor responder groups, with the exception of AcSP.

![Fig. 1.—Neutrophil superoxide generation during SASP treatment (plateau levels).](image-url)
levels at week 16, these being higher in those patients showing a poor response \( (P = 0.036)\).

**DISCUSSION**

Despite SASP’s proven anti-rheumatoid activity, only between 29 and 34% of patients remain on treatment after 4–5 yr. Of the two-thirds of patients failing to continue treatment, approximately half withdraw due to lack of efficacy and half due to toxicity. The most common side-effects are gastrointestinal, primarily nausea and vomiting, although others include skin rash, mouth ulcers, neutropenia, dizziness, megaloblastic anaemia, hepatitis and reversible oligospermia [23].

Clearly, prior knowledge of those patients likely to find SASP efficacious would enable better targeting of drug therapy and, in addition, could provide information towards the elucidation of the mode of action of the drug. Previous work in our unit has shown an inverse correlation between the response to SASP and serum antibody levels to antigens of *C. perfringens*, suggesting that immune mechanisms may be involved [11]. This observation prompted us to investigate other aspects of the immune response, namely SASP/SP on neutrophil superoxide production, in good and poor responders to SASP to establish whether this also reflected the likelihood of response to this drug.

Our study showed no difference in superoxide production between responders and non-responders to SASP or normal controls. Neutrophil superoxide production, as measured by plateau levels, was, however, shown to decrease significantly in responders but not in non-responders to SASP. Initial activity rate levels also fell in the responder group, but this did not reach significance. However, our data came from only a small group of patients and investigation of a larger

<table>
<thead>
<tr>
<th>Table III</th>
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<tr>
<td>Effect of sulphasalazine and sulphapyridine on neutrophil superoxide production <em>in vitro</em></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Plateau levels</th>
<th>Initial rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ( \mu )M</td>
<td>100 ( \mu )M</td>
</tr>
<tr>
<td>Good responders</td>
<td>58.9</td>
<td>73.2</td>
</tr>
<tr>
<td>Poor responders</td>
<td>56.9</td>
<td>74.6</td>
</tr>
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</table>

Results are expressed as the percentage of neutrophil superoxide production in the absence of drug.
group of patients may show this change to be of significance.

The effect on superoxide production observed could be due to one of three reasons. First, it could be due to a differential action by SASP/SP on neutrophils in patients who respond compared to those patients who failed to respond. Second, it could be due to higher circulating drug levels in those patients who responded, although serum levels of SASP and its major metabolites have been found to be unrelated to response [5]. Third, it could be an effect secondary to a general downgrading of the immune response brought about by some other mechanism.

A difference in the susceptibility of neutrophils to the action of SASP/SP in responders and non-responders might cause differences in the way in which the cells are affected by these drugs in vitro. Our study showed no difference in the way in which neutrophils from the two groups of patients reacted to the addition of SASP/SP in vitro. Also, plasma levels of SASP, SP and AcSP were unrelated to the response to drug, suggesting that there is no inherent difference in the susceptibility of neutrophils in responders and non-responders to SASP.

This suggests that SASP’s action on neutrophils is not the main mechanism of its mode of action in RA. It also indicates that neutrophil superoxide production is not a suitable marker for therapeutic targeting of SASP in RA, although whether other markers might exist requires further investigation, as does our earlier observation of altered antibody responses to bacterial antigens.

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REFERENCES